How do two unrelated antibodies, HyHEL-10 and F9.13.7, recognize the same epitope of hen egg-white lysozyme?

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Abstract

The anti-hen egg-white lysozyme (HEWL) antibodies HyHEL-10 and F9.13.7 recognize a common epitope. The structures of the complexes differ, however, in the numbers of electrostatic and hydrogen-bond interactions and in the distributions of contacts between the light and heavy chains. The equilibria and kinetics characterizing the F9.13.7 complex formation were evaluated for both wild-type and mutant derivatives of HEWL to help to understand how the different contacts are effectively used in the complexes with the two antibodies. Three epitope hot spots, Y20, K96, and R73 (destabilization > 4 kcal/mole), were found by alanine scanning mutagenesis. The first two constitute two of the three hot spots in the HyHEL-10 complex. The hot spots of the HyHEL-10 paratope are centered on the HEWL epitope; whereas R73 (HEWL), the only important light-chain-contacting residue, is clearly separated from the other hot spots of the F9.13.7 complex. The larger number of epitope warm plus hot spots found in the F9.13.7 complex compared with that of HyHEL-10 shows that the specificity of the former is greater even though the K_D value is 20-fold larger. Conservative mutations showed that the specificity enhancement is related to the greater number of functional polar and hydrogen bond interactions in the F9.13.7 complex. Alanine scanning mutagenesis would not have illuminated these distinctions. It is shown that the concept of antigen specificity, as defined by cross-reactivity with natural variant antigens, is flawed by phylogenetic bias, and that specificity can only be defined by the use of unbiased epitopes, which are conveniently accessed by site-directed mutagenesis.

Keywords: Alanine scanning mutagenesis; antigen; antibody; epitope mapping; hen egg-white lysozyme; scFv HyHEL-10; scFv F9.13.7; protein–protein interaction

The vast antibody recognition repertoire, dictated by the six small hypervariable regions (complementarity-determining regions, CDRs), is responsible for the combination of exquisite specificity of mature antibodies and the thermody-

namic stability of the antigen–antibody complexes (Chothia et al. 1989; Padlan 1990). Crystallographically defined complexes of antibodies with protein antigens identify putatively important partner interactions in the interface (Amit et al. 1986; Chitarra et al. 1993; Braden and Poljak 1995; Davies and Cohen 1996). Although such structural data do localize these specific interactions topologically, they do not address the specificity and thermodynamic questions. Recent approaches to these topics have focused on investigations of the interactions of a single antibody with unrelated antigens (Dall'Acqua et al. 1996) and of the refinement of specificity with maturation (Goldbaum et al. 1999; Lavoie et al. 1999).

Can a given epitope drive the formation of multiple paratopes of unrelated sequence? The answer appears to be yes,

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Abbreviations: CDRs, complementarity-determining regions; Fab, antigen-binding fragment; GEL, guinea fowl lysozyme; HEWL, hen (chicken) egg-white lysozyme; HyHEL-10 and F9.13.7, monoclonal antibodies raised against HEWL; scFv, single-chain variable fragment; subscripts L and H, antibody light and heavy chain, respectively; WT, wild type.

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because the HyHEL-10/lysozyme epitope is also recognized by the F9.13.7 Fab (Lescar et al. 1995). HyHEL-10 and F9.13.7 are two independently isolated high-affinity antibodies that bind nearly the same epitope of hen egg-white lysozyme (HEWL), but they have different, nonhomologous, CDRs.

The crystallographic structures of HyHEL-10 in complex with HEWL (Padlan et al. 1989; Kondo et al. 1999) and F9.13.7 with guinea fowl lysozyme (GEL; Lescar et al. 1995) show that they interact with essentially the same conserved epitope residues (Table 1). The buried surface area totals $\sim 1600 \text{ \AA}^2$ in both complexes. This contacting area is nearly equally shared between the heavy and light chains of HyHEL-10; however, it is unevenly distributed for F9.13.7 ($570 \text{ \AA}^2$ for the heavy and $220 \text{ \AA}^2$ for the light chain). The HyHEL-10 complex features 14 intermolecular hydrogen bonds and one salt bridge, whereas the F9.13.7 complex contains 12 hydrogen bonds and three salt bridges. The polar and charged groups are distributed similarly in the two antibody combining sites. These antibodies show different cross-reactivity patterns with related avian lysozymes (Smith-Gill et al. 1984; Tello et al. 1990). Association of either antibody with HEWL inactivates the enzyme by insertion of a CDR loop into the active site.

The HyHEL-10/HEWL interaction has been extensively examined. Site-directed mutagenesis studies have quantified the contribution of the epitope (Kam-Morgan et al. 1993; Rajpal et al. 1998; Rajpal and Kirsch 2000) and paratope (Tsumoto et al. 1995, 1996; Pons et al. 1999) residues, and the results have been compared with computed experimental results (Pomès et al. 1995; Sharp 1998). The effects of natural or in vitro affinity maturation have been studied thermodynamically (Nishimiya et al. 2000) and kinetically (Lavoie et al. 1999). The differential effects of HEWL mutations on association and disassociation rate constants led to the development of a general method to delineate docking trajectories in protein-protein interactions (Taylor et al. 1998).

The F9.13.7/lysozyme interaction, by comparison, is largely unexplored. No systematic site-directed mutagenesis study has focused on this complex. The above-mentioned cross-reactivity results, the crystal structure, and a thermodynamic comparison of the energetics of binding of F9.13.7 and other specific antibodies with lysozyme constitute what is known about this complex.

We report here the construction and expression of a synthetic *scFv* gene for F9.13.7, the quantitative analysis of the contribution of epitope residues to the stability of the com-

Table 1. Effect of mutations in the common HEWL epitope on free energies of association with F9.13.7- and HyHEL-10-*scFv*

HEWL mutant	F9.13.7/HEWL K_D (nM) ^a	F9.13.7/HEWL $\Delta\Delta G$ (kcal/mole) ^b	HyHEL-10/HEWL $\Delta\Delta G$ (kcal/mole) ^b
WT	0.57 $\pm$ 0.05		(K_D 0.03 $\pm$ 0.001 nM)
Alanine mutations			
R14A	3 $\pm$ 1	0.9 $\pm$ 0.2	No contact
H15A	18 $\pm$ 1	2.03 $\pm$ 0.07	-0.44 $\pm$ 0.07
Y20A	50,000 $\pm$ 1000	5.38 $\pm$ 0.15	4.9 $\pm$ 0.1
R21A	140 $\pm$ 15	3.26 $\pm$ 0.08	1.07 $\pm$ 0.07
W63A	11 $\pm$ 1	1.76 $\pm$ 0.07	0.3 $\pm$ 0.2
R73A	106,000 $\pm$ 80,000	7.2 $\pm$ 0.9	-0.3 $\pm$ 0.1
L75A		No contact	1.24 $\pm$ 0.04
N77A	1.5 $\pm$ 0.2	0.57 $\pm$ 0.09	No contact
T89A		No contact	0.01 $\pm$ 0.05
N93A	0.24 $\pm$ 0.07	-0.5 $\pm$ 0.2	0.6 $\pm$ 0.1
K96A	1000 $\pm$ 200	4.4 $\pm$ 0.1	7.0 $\pm$ 0.3
K97A	150 $\pm$ 13	3.29 $\pm$ 0.07	6.2 $\pm$ 0.1
I98A		No contact	-0.03 $\pm$ 0.07
S100A	4.9 $\pm$ 0.4	1.26 $\pm$ 0.07	0.26 $\pm$ 0.08
D101A	60 $\pm$ 10	2.7 $\pm$ 0.1	1.5 $\pm$ 0.1
Conservative mutations			
Y20F	220 $\pm$ 30	3.59 $\pm$ 0.09	0.06 $\pm$ 0.03
Y20L	3,600 $\pm$ 600	5.2 $\pm$ 0.1	2.7 $\pm$ 0.1
R73M	ND	>8	-0.26 $\pm$ 0.04
K96M	17,000 $\pm$ 10,000	6.1 $\pm$ 0.6	7.6 $\pm$ 0.1
K97M	240 $\pm$ 10	3.58 $\pm$ 0.06	1.1 $\pm$ 0.1
K97R	1800 $\pm$ 300	4.7 $\pm$ 0.1	3.6 $\pm$ 0.3

^a K_D values were determined as described in Materials and Methods.

^b $\Delta\Delta G = RT \ln(K_{D(\text{mut-complex})}/K_{D(\text{WT-complex})})$.

plex by both alanine scanning mutagenesis and conservative mutations, and a comparison with the HyHEL-10/HEWL complex. These two structurally and sequence diverse antibodies, which bind to the same epitope, offer a unique probe to explore multiple modes of protein–protein recognition and to elucidate further relationships between affinity and specificity.

Results and Discussion

Comparison of F9.13.7 scFv and Fab

The three-dimensional structure of the F9.13.7–lysozyme complex (Lescar et al. 1995) shows that CDR-H3 protrudes into and partially fills the substrate-binding cleft of lysozyme, thereby inhibiting enzyme activity. This feature allows convenient monitoring of the extent of association of the two proteins by homogeneous solution kinetic assays developed earlier in this laboratory (Rajpal et al. 1998; Taylor et al. 1998). To compare the functional characteristics of the newly constructed scFv protein with the Fab fragment of the natural antibody, kinetic studies were performed in tandem with both proteins. The k_{on} values are very similar (scFv: $1.36 \pm 0.08 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$; Fab: $1.4 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$). The k_{off} values are ~ 2.8 times greater for the Fab complex with lysozyme (scFv: $8.0 \pm 0.5 \times 10^{-4} \text{ sec}^{-1}$; Fab: $22 \pm 2 \times 10^{-4} \text{ sec}^{-1}$). The calculated K_D values (scFv: $0.57 \pm 0.05 \text{ nM}$; Fab: $1.6 \pm 0.2 \text{ nM}$) show that scFv binds ~ 2.5 times more tightly to HEWL than does the corresponding Fab. These results show that the synthesized scFv is fully functional. Increased affinity of scFv over Fab has been reported elsewhere (Iliades et al. 1998). The K_D values obtained here by homogeneous kinetics are very close to the results obtained with the F9.13.7 Fab by calorimetry ($1.3 \pm 0.6 \text{ nM}$; Schwarz et al. 1995).

Alanine scanning mutagenesis of F9.13.7

The epitope of the GEL–Fab F9.13.7 complex is identified in the crystal structure (Lescar et al. 1995). The guinea fowl and chicken lysozymes differ by only 11 amino acids in sequence; however, all residues in the epitopes recognized by HyHEL-10 and F9.13.7 are conserved. The RMSDs for C_α atoms in equivalent positions are $<0.9 \text{ \AA}$. All non-glycine HEWL residues in contact with F9.13.7 in the complex were individually replaced with alanine for comparison with the previously obtained data with the HyHEL-10 Fab (Rajpal et al. 1998) and HyHEL-10 scFv (this work). The K_D and $\Delta\Delta G$ values are collected in Table 1.

The mutations Y20A, R73A, and K96A each destabilizes the complex by $>4 \text{ kcal/mole}$. Thus residues Y20, R73, and K96 are defined as hot spots. Both Y20 (OH) and K96 (NH_3^+) interact with the carboxyl group of D52_H in the CDR-H2 loop, forming a hydrogen bond and a salt bridge,

respectively. Although most of the important F9.13.7 interactions with HEWL involve the heavy chain, there is a single significant contact residue in HEWL that is made with the F9.13.7 light chain. Mutation of R73, which makes hydrogen bonds with (Y32_L) in CDR-L1 and Y92_L in CDR-L3, causes the largest destabilization effected by alanine substitution ($\Delta\Delta G = 7.0 \text{ kcal/mole}$).

Moderate destabilization, $1 < \Delta\Delta G < 4 \text{ kcal/mole}$ (warm spots), is seen in complexes formed from HEWLs with alanine mutations at H15, R21, W63, K97, S100, and D101. H15 and K97 form the two remaining salt bridges of the complex, H15/D54_H and K97/E50_H, respectively. The W63 side chain only contacts the antibody through CD2 of T104_H and the S105_H backbone; however, the indole group makes close contacts with the hot spot K97 as well as with epitope residues W62 and L75, which may buttress R73. The same role has been attributed to some of the HyHEL-10 epitope warm spots (Rajpal and Kirsch 2000). Thus the effect of the W63A mutation may be on the lysozyme structure itself. The R21, S100, and D101 residues each contributes hydrogen bonds to the CDR-H3 loop: that is, to Y96_H, T100_H, and S100(A)_H, respectively. The remaining contact residues in the crystal structure, R14, N77, and N93, destabilize the complex by $<1 \text{ kcal/mole}$ when mutated to alanine, and are thus null spots.

F9.13.7 versus HyHEL-10: Conservation of residues

The relative importance of alanine epitope mutations for both complexes is diagrammed in Figure 1. All three epitope hot-spot residues in the HyHEL-10/HEWL complex (Y20, K96, and K97) are also important in the corresponding F9.13.7 complex. R73 provides an additional hot spot in the latter complex, but contributes negligibly to the stability of the HyHEL-10/HEWL complex. The definition of largely

Fig. 1. Comparison of the effects of mutations to alanine in the HEWL epitope on the destabilization of the F9.13.7 (shaded bars) and HyHEL-10 (speckled bars) lysozyme complexes.

coincident hot spots for the two complexes indicates that certain residues/features are strongly preferred for antibody recognition within a given epitope.

Two of the three HEWL warm-spot residues in the HyHEL-10/HEWL complex are also warm spots, as defined above, in the F9.13.7 complex, but they show a somewhat greater destabilization when mutated to alanine in the latter complex (R21: 1.0 vs. 3.2 kcal/mole; D101: 1.5 vs. 2.7 kcal/mole for HyHEL-10 and F9.13.7, respectively). L75, the third warm spot of HEWL in the HyHEL-10 complex does not interact with F9.13.7. The F9.13.7/HEWL complex has four additional warm spots, K97, H15, W63, and S100. K97, a hot spot for HyHEL-10/HEWL, is the most important F9.13.7 warm spot and destabilizes the complex by 3.3 kcal/mole. H15, W63, and S100 are null spots in the interaction with HyHEL-10. The interaction between F9.13.7 and HEWL has more warm spots, and those that are shared in the two antibody complexes cause greater destabilization when mutated to alanine in F9.13.7/HEWL than the same mutations in the HyHEL-10/HEWL complex.

The relative ranking for the alanine scanning destabilizations of the two complexes is F9.13.7: R73A > Y20A > K96A > K97A = R21A > D101A > H15A > W63A > S100A. HyHEL-10: K96A > K97A > Y20A $\gg$ D101A > L75A > R21A (see Table 1). The positions of the HEWL hot-, warm-, and null-spot residues in both antibody complexes are shown in Figure 2. Although the important residues are essentially the same in both complexes, the topologies differ. In HyHEL-10 there is a clear center of interaction with its three hot spots (Y20, K96, and K97), whereas in F9.13.7 the hot spot R73 is separated from the other hot spots, Y20 and K96, by

a patch of warm spots. The percentages of warm plus hot spots in the epitopes are 75% and 46% in the F9.13.7 and HyHEL-10 complexes, respectively.

Affinity versus specificity

The higher number of hot and warm spots in the interaction with F9.13.7/HEWL is clearly seen in Figure 3, which shows the losses in free energies of formation in HyHEL-10/HEWL complexes affected by epitope alanine substitutions compared with the effects in the F9.13.7/HEWL complexes. The mutations that fall on the line of unit slope are equally important in determining the affinity in both complexes. Those that are found in the upper, dark-shaded triangle are more important for the interaction with HyHEL-10, and those located in the lower triangle more significantly destabilize the F9.13.7/HEWL complex. This plot does not include the contributions of L75, R14, and N77, which are not common to both complexes (see Table 1). What is meant by specificity? It may be considered a function of an antibody's power of discrimination between similar antigens. Quantitatively, a specificity determinant may be defined by eliciting a $\Delta\Delta G$ for association with an antigen that is greater than an arbitrary threshold level in response to a given replacement of that determinant in the antigen. The more specific antibody will show such a response $\Delta\Delta G$ for a larger number of amino acid substitutions in the antigen. As documented above, there are a significantly greater number of important energetic determinants in the F9.13.7 than in the HyHEL-10/HEWL complex; therefore, F9.13.7 shows greater specificity despite its 20-

Fig. 2. Ribbons diagram (Carson 1997) of contact residues in the HEWL epitope for HyHEL-10 (*left*) and F9.13.7 (*right*) paratope residues. Amino acids whose mutation to alanine gives a $\Delta\Delta G > 4$ kcal/mole (hot spots) are shown in red, those yielding 1 kcal/mole $< \Delta\Delta G < 4$ kcal/mole (warm spots) are in yellow, and those with $\Delta\Delta G < 1$ kcal/mole are in blue (null spots).

Fig. 3. Free energies of destabilization effected by epitope alanine mutations in lysozyme complexes with HyHEL-10 compared with those for the same mutations in the F9.13.7 complexes. Those residues shown above the line of unit slope contribute more significantly to the stability of the HyHEL-10 complexes, whereas those beneath the line are more important for the stability of the F9.13.7 complexes.

fold lower affinity for HEWL (K_D for HEWL/HyHEL-10 = 30 pM; K_D for HEWL/F9.13.7 = 600 pM).

To define further the specificity differences between the two antibodies, additional conservative mutations were made in the residues defined as hot spots for both complexes. Figure 4 compares the $\Delta\Delta G$ with respect to the wild-type complex for each of these mutants. These results emphasize the necessity of including mutations in addition to alanine to illuminate fully the details of the contributed interactions.

Fig. 4. Histogram showing the specific destabilization free energies for HyHEL-10 (speckled bars) and F9.13.7 (shaded bars) complexes effected by alanine and conservative mutations in the lysozyme epitope.

The Y20A mutation destabilizes both antibody/HEWL complexes by >4 kcal/mole. The Y20F mutation shows a major difference in the interaction of Y20 with the two different antibodies. Removal of the hydroxyl group from the phenyl ring has virtually no effect on the K_D value for the HyHEL-10/HEWL complex ($\Delta\Delta G = 0.06 \pm 0.03$ kcal/mole), indicating that all of the important contacts with this residue are located in the ring. However, this conservative mutation is strongly disruptive in the F9.13.7/HEWL complex (3.6 ± 0.09 kcal/mole). These large F9.13.7 complex destabilizations correspond to the loss of the hydrogen bond between the Y20 (O_η) and D52_H ($O_{\delta 1}$). The semiconservative mutation Y20L, which presumably retains an intermediate amount of hydrophobic contacts (intermediate between alanine and phenylalanine), shows a moderate destabilization value (2.7 ± 0.1 kcal/mole) for the interaction with HyHEL-10. This mutation also yields a major destabilization of the F9.13.7 complex (5.2 ± 0.1 kcal/mole).

The K96M mutation results in a >4 kcal/mole destabilization of both antibody complexes. These values are similar to those observed in the K96A complexes; therefore, the important interaction is due almost entirely to the K96 ϵ amino group, which forms a hydrogen bond with N31_L (HyHEL-10) or a salt bridge with D52_H ($O_{\delta 2}$) (F9.13.7). The hydrogen bond K96/N31_L contributes 5.2 kcal/mole to the stability of the HyHEL-10 complex (Pons et al. 1999). The present results show that the salt bridge K96/D52_H is also very important for the interaction between F9.13.7 and HEWL.

Both antibodies form salt bridges with HEWL at position K97 (HyHEL-10, K97/D32_H and F9.13.7, K97/E50_H). K97 mutations in lysozyme show different effects on the stabilization of the two antibody complexes. K97A shows a two-fold greater free energy of destabilization of the HyHEL-10 complex, compared with the K97A/F9.13.7 complex (6.2 vs. 3.3 kcal/mole, respectively). The K97M mutation restores most of the lost interaction in the K97A complex with HyHEL-10 (1.1 kcal/mole), but equally destabilizes the F9.13.7 complex (3.6 kcal/mole). These data show that the side-chain interactions of K97 in the HyHEL-10 complex are more important than the salt bridge, but the converse is true for the F9.13.7 complex. It has been shown that the entire salt bridge can be replaced by chain-length conservative mutations in the HyHEL-10 interaction (K97M/D32_HN) with a very small destabilization, 0.3 ± 0.1 kcal/mole (Pons et al. 1999).

The R73 mutations are silent in their effects on the stabilities of the HyHEL-10 complexes (-0.3 kcal/mole); however, they define R73 as the most important residue for the stabilities of the F9.13.7 complexes (>7 kcal/mole). The guanidino nitrogen atoms participate in three hydrogen bonds: R73 (N_ϵ)/Y92_L (O); R73 (N_{H1})/S100_H(A) (O); and R73 (N_{H2})/Y32_L (O_η). The fact that the R73A and R73M mutations are equally destabilizing isolates the guanidino moiety as the major contributing factor.

Polar interactions are more important for the higher-specificity antibody

There are more energetically important polar contacts observed in the F9.13.7 complex than in that formed with HyHEL-10. Conversely, two out of three HEWL hot spots interact mainly by side-chain van der Waals contacts in the HyHEL-10 complex. Although about half of the amino acids found in proteins have side chains with the potential to participate in hydrogen bonds or salt bridges, such interactions require specific complementary functional groups and geometrically constrained participation from the associating partner protein. Nonpolar interactions are not similarly constricted. Thus, few replacements will maintain polar interactions, whereas a greater number of substitutions are allowed to maintain the strength of a nonpolar interaction. And therefore, antibodies that obtain most of the complex stabilization energy from nonpolar interactions will be less specific than antibodies with a high number of significant polar contacts. The same relation between nonpolar interactions and cross-reactivity have been described in receptor-hormone and other protein-protein interactions (DeLano et al. 2000).

How is specificity defined in antibody-antigen interactions

An antibody is generally considered specific if it cross-reacts with only a few natural variants of the antigen. The lysozyme antibody literature provides a rich data source for comparative purposes. To date, most specificity studies have focused on cross-reactivity reactions of antibodies with closely related natural antigens. The results of these investigations may, however, suffer from bias owing to the phylogenetic variability of the epitope. That is, antibodies directed to conserved epitopes will be highly cross-reactive, and are therefore categorized as nonspecific, whereas those targeted to variable epitopes will not cross-react, and are thus termed specific. Thus, specificity as so defined does not address the intrinsic capacity of different antibodies to detect possible variations in the antigen. It follows that investigation of cross-reactivity with naturally occurring variants will not serve to define the potential specificity of antibodies properly. To illustrate, D1.3 and D11.15 are two highly affine monoclonal antibodies raised against HEWL from the same mouse. The epitopes of these two antibodies partially overlap, but each shows a different cross-reactivity pattern. D1.3 cross-reacts only with bobwhite quail lysozyme and, therefore, has been termed a highly specific antibody, whereas D11.15 cross-reacts with all the tested avian lysozymes (Harper et al. 1987) and was categorized as a nonspecific antibody. The crystal structures of D1.3 complexed with HEWL (Amit et al. 1986; Fischmann et al. 1991) and D11.15 complexed with pheasant egg lysozyme

(Chitarra et al. 1993) explain the differences in cross-reactivity patterns between these closely related antibodies. Q121, which is present only in chicken and bob white quail lysozymes, is an essential component of the D1.3 epitope. On the other hand, the centrally located residues in the D11.15 epitope are strictly conserved among all avian lysozymes. Therefore, the observed difference in cross-reactivity between these antibodies is the result of fortuitous variability of the natural antigens. For example, just the opposite specificity classification would have been concluded if lysozyme Q121 were conserved and the essential residues for the interaction with D11.15 were variable among avian lysozymes.

The preceding analysis shows the necessity to investigate unbiased epitopes, which are accessible only by site-directed mutagenesis, to quantify antibody specificity. Alanine scanning mutagenesis is a method for systematic screening of the entire epitope. We have shown, through the application of alanine scanning mutagenesis of a system where two antibodies bind the same structural epitope, that antibodies with lower affinity for one epitope can at the same time detect more variations and therefore be more specific. This shows that affinity and specificity will not always correlate.

Materials and methods

The materials and procedures not described here are found in Rajpal and Kirsch (2000). The F9.13.7 Fab was a gift from R.J. Poljak.

F9.13.7 scFv gene synthesis

An scFv gene with the identical variable domain sequences of the crystallized Fab of F9.13.7 (Lescar et al. 1995) was constructed by sequential subcloning of nine small fragments (~160 bp) into the *Pichia pastoris* pPIC-9 plasmid (Invitrogen). Each fragment was generated by PCR fill-in of both overhang single-stranded portions of two large oligonucleotides (~90 bp) that were annealed together with a complementary region (~25 bp) located at the 3' end of the top oligonucleotide and the 5' end of the bottom oligonucleotide. The final gene contains in the following order from the N terminus: (1) the heavy-chain variable domain (V_H), (2) a linker sequence (SSASSGGGGSGGGSGGGGS), (3) the light-chain variable domain (V_L of the κ family), and (4) a penta-his tag.

The final F9.13.7 scFv protein sequence is: VSLEKRQVQLQQPGAELVKPGASVKLSCKASGYTFTSYWMHWVKQPGQGLEWIGEIDPSDYPNYNEKFKGKATLTVDKSSSTAYMQLSSLTSEDSAVYYCASLYYYGTSYGVLIDYWGQGTSTVTVSSASSGGGGSGGGSGGGSDIQMTQTSSLSASLGDRVTISCRASQDISNYLNWYQKKPDGTVKLLIYYTSLRHSGVPSRFSGSGSGTDYSLTIRNLEQEDIATYFCQQGYTLPTFGGGTKLEIKRHHHHH.

Expression and purification

The scFvs were purified as described in Pons et al. (1999), and HEWL as described in Matsumura and Kirsch (1996). The purities

of the samples were verified by SDS-PAGE. The absence of glycosylation for all the proteins and the correct mass for mutants was confirmed by electrospray mass spectrometry (facility of the Department of Chemistry, University of California, Berkeley). No dimers of scFvs were detected by HPLC gel filtration (Bio-sil-sec 250 Column, Bio-Rad).

Site-directed mutagenesis

Some HEWL mutant genes were available from previous investigations of this laboratory (Rajpal et al. 1998; Taylor et al. 1998). Additional mutations were produced by PCR with either the mega-primer method (Landt et al. 1990; Pons et al. 1997) or by recombinant PCR using two mutagenic oligonucleotides (Innis et al. 1995). All mutants were fully sequenced.

Determination of kinetic parameters for complex formation and dissociation

Both HyHEL-10 and F9.13.7 occlude the active site of HEWL. The complex retains only 5% of the catalytic activity of free HEWL; therefore, HyHEL-10 binding with HEWL can be determined in homogeneous solution by monitoring the rate of loss of activity of HEWL following addition of scFv as described by Taylor et al. (1998). The values of k_{on} were determined from nonlinear regression of the data using:

$$Abs_{obs} = Abs_{init} + v_{bl} \cdot t - \left[\frac{k_{cat} \cdot [E_T] \cdot [S]}{k_{on} \cdot K_m \cdot [Ab_T]} \cdot e^{(k_{on} \cdot [Ab_T] \cdot K_m \cdot t / K_m + [S])} \right],$$

where Abs_{init} is the initial absorbance at 450 nm, v_{bl} is the settling rate of the substrate cell-wall particles in the absence of HEWL, $[E_T]$ is the total concentration of HEWL, $[Ab_T]$ is the total scFv concentration, and $[S]$ is the initial substrate *Micrococcus luteus* cell-wall concentration. Only those fits to the preceding equation yielding a correlation coefficient $R \geq 0.995$ were accepted.

The dissociation rate constants, k_{off} , were determined by monitoring the rate of recovery of active HEWL from a preformed scFv/HEWL complex. The nascent free antibody was sequestered with an excess of E35Q HEWL, an inactive mutant. The data were fitted to the following equation (Rajpal et al. 1998),

$$v_{obs} = k_{cat} \cdot [E_T] \cdot (1 - e^{-k_{off}t}) + v_{init} \cdot (e^{-k_{off}t}) + v_{bl},$$

where v_{init} is the enzymatic activity due to the complex and trace amounts of HEWL before the addition of E35Q HEWL. The kinetic assays were performed at pH 7.0 instead of pH 6.2 (Pons et al. 1999; Rajpal and Kirsch 2000) to eliminate scFv-10 precipitation (pI = 6.0, as calculated from the amino acid composition). The k_{on} and k_{off} values for the formation and dissociation of the wild-type HEWL-scFv complex at pH 7.0 were similar to those obtained at pH 6.2 (data not shown). All data were obtained on a Uvikon 860 (Kontron Instruments) spectrophotometer and fitted by nonlinear regression to their respective models using the application Kaleidagraph (Synergy Software).

The kinetics are too fast to monitor by the above methods, where K_D values are >3 nM ($100 \times K_D$ WT); therefore, only the values of K_D determined by equilibrium methods (Kam-Morgan et al. 1993) are given.

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